A novel family of fluorescent hypoxia sensors reveal strong heterogeneity in tumor hypoxia at the cellular level

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Abstract

Hypoxia is an intensively investigated condition with profound effects on cell metabolism, migration, and angiogenesis during development and disease. Physiologically, hypoxia is linked to tissue homeostasis and maintenance of pluripotency. Hypoxia also contributes to pathologies including cardiovascular diseases and cancer. Despite its importance, microscopic visualization of hypoxia is largely restricted to the detection of reductively activated probes by immunostaining. Here, we describe a novel family of genetically encoded fluorescent sensors that detect the activation of HIF transcription factors reported by the oxygen-independent fluorescent protein UnaG. It comprises sensors with different switching and memory behavior and combination sensors that allow the distinction of hypoxic and reoxygenated cells. We tested these sensors on orthotopically transplanted glioma cell lines. Using a cranial window, we could visualize hypoxia intravitaly at cellular resolution. In tissue samples, sensor activity was detected in regions, which were largely devoid of blood vessels, correlated with HIF-1α stabilization, and were highly heterogeneous at a cellular level. Frequently, we detected recently reoxygenated cells outside hypoxic areas in the proximity of blood vessels, suggestive of hypoxia-promoted cell migration.

Keywords fluorescent protein; hypoxia; microscopy; sensor; UnaG

Subject Categories Vascular Biology & Angiogenesis

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Introduction

Hypoxia, a dysbalance between oxygen supply and consumption, plays a crucial role in developmental, regenerative, and pathological processes (Span & Bussink, 2015). A hypoxic niche supports pluripotency of stem cells in bone marrow (Mathieu et al., 2013; Muz et al., 2014; Spencer et al., 2014), and hypoxia causes the recruitment of inflammatory cells, promotes the progression of tumors to a more aggressive phenotype, mediates radio resistance, and enhances metastatic spread (Hockel & Vaupel, 2001; Chan & Giaccia, 2007; Semenza, 2012).

While incipient angiogenesis in small tumors may occur independently of hypoxia (Cao et al., 2005), growing tumors will at some point inevitably experience inadequate nutrient and oxygen supply (Hendriksen et al., 2009). This deprivation triggers an angiogenic switch, which is associated with reduced sensitivity to cytotoxic and genotoxic treatment and more aggressive metastatic behavior (Hanahan & Folkman, 1996). Therefore, precise knowledge of the hypoxic state of a tumor not only provides a valuable entry point to understanding tumor progression, but also holds considerable prognostic value (Bussink et al., 2003).

A collection of techniques are available to assess hypoxia in living tissues or tissue preparations (Kiyose et al., 2010; Cui et al., 2011). Assays that rely on tissue disaggregation or invasive polargraphic needle and fluorescence-based fiber-optic probe measurements suffer from their inability to provide information on hypoxia and tissue architecture (Raleigh et al., 1996). When applied \textit{in vivo}, nitroimidazole derivatives in the absence of oxygen reductively form tissue adducts (Kizaka-Kondoh & Konse-Nagasawa, 2009). For optical imaging, these adducts are stained with monoclonal antibodies postmortem. Labeling of hypoxia-targeting molecules with positron-emitting radionuclides allows non-invasive live imaging, which provides the spatial distribution of the probe, however not at cellular resolution (Apte et al., 2011). Intravital determination of the partial oxygen pressure in the microvasculature has been achieved using multiphoton enhanced phosphorescent nanoprobes (Lecoq et al., 2011; Roussakis et al., 2014; Spencer et al., 2014). However, these rely on tissue perfusion as they are systemically applied via the circulation.

Central to the cellular hypoxia response is the hypoxia-inducible factor (HIF) family of basic helix-loop-helix transcription factors that act on a wide array of hypoxia-inducible genes (Fandrey et al., 2006; Semenza, 2011). HIFs act as heterodimers and are comprised of the oxygen-independent β-subunit (also called aryl hydrocarbon receptor nuclear translocator, ARNT) and an oxygen-labile α-subunit,
which is rapidly degraded under normoxia or upon reoxygenation from a previously hypoxic state (Salceda & Caro, 1997). Proteasomal degradation is triggered by hydroxylation of two proline residues, which provides docking sites for the von Hippel–Lindau (VHL) tumor suppressor. E3 ubiquitin ligase (Srinivas et al., 1999). The Fe (II)-dependent HIF prolyl hydroxylases (PHD1-3) are efficiently inhibited by transition metals such as Co⁷⁺ or iron chelators, and their target prolines are central to the oxygen-dependent degradation domain (ODDD) in HIF-α (Yuan et al., 2003; Fandrey et al., 2006; Cho et al., 2013). Additional hydroxylation of an Asn residue in the transactivation domain by the factor inhibiting HIF (FIH) results in suppressed transcriptional activity (Lando et al., 2002).

Abating PHD and FIH activity due to reduced O₂ tension results in HIF-α stabilization, translocation into the nucleus, and binding to HIF-β. The HIF-α/β heterodimer then activates transcription from promotor regions of genes that contain hypoxia-responsive elements (HREs: 5'-RCGTG-3' with R=A,G), including the genes for vascular endothelial growth factor (VEGF-A), carbonic anhydrase IX (CA9), the glucose transporters (Glut-1 and Glut-3), and erythropoietin (Wenger et al., 2005).

Given the existence of positively hypoxia-responsive promoter elements, but also specific oxygen-dependent destruction domains, it should be possible to design genetically encoded hypoxia reporters, with the promise of tissue-wide hypoxia visualization at cellular resolution (Harada et al., 2007; Fomicheva et al., 2008). While seemingly straightforward, a combination of these regulatory elements with fluorescent GFP or RFP proteins is ill-fated, because both of these protein families must undergo an essential maturation process comprising torsional rearrangement, cyclization, and oxidation that requires molecular oxygen, to adopt their fluorescent state (Heim et al., 1994; Coralli et al., 2001; Shaner et al., 2005; Remington, 2006; Cecic et al., 2007).

Here, we describe a novel family of genetically encoded hypoxia sensors, which are based on a recently identified, fluorescent protein from Japanese freshwater eel, UnaG (Kumagai et al., 2013). In contrast to the GFP and RFP fluorescent protein families, UnaG does not rely on molecular oxygen to adopt the fluorescent state. In these sensors, the combination of genetically and protein-encoded regulatory elements confers hypoxia-mediated expression. Through constitutive or hypoxia-mediated degradation sensitivity, memory and switching behavior of these reporter constructs were tuned to suit various intravital applications. Further, the combination of oxygen-sensitive fluorescent proteins with UnaG results in hypoxia and reoxygenation reporters that unequivocally document the recent hypoxia history of cells in vivo in a dynamic fashion.

Results

UnaG-based sensors allow efficient hypoxia sensing at cellular level

To avoid the limitations imposed by oxygen-dependent maturation of GFP and RFP, we designed a UnaG-based, genetically encoded hypoxia sensor for light microscopy (Fig 1A), which uses an established hypoxia-responsive promoter (Semenza et al., 1991). Five copies of a 35-bp hypoxia-responsive element (HRE) derived from the enhancer region of the human VEGFA gene (Shibata et al., 2000) confer oxygen sensitivity to a minimal human cytomegalovirus (CMV) promoter, which drives UnaG expression. To enhance protein turnover and hence dynamic behavior, a PEST (Rogers et al., 1986; Li et al., 1998) sequence motif was fused in-frame to the C-terminus of UnaG. All constructs in this study share the same vector backbone. For simplicity, we refer to them by a combination of the fluorescent protein name and the destabilization sequence (except for the hypoxia–reoxygenation reporter dUnOHR).

We tested the efficiency of the UnaG-based sensor by comparing it to an available variant, which in the same vector backbone harbors dEGFP instead of dUnaG (Vordermark et al., 2001). To avoid the effects of clonal variation, we used CHO bulk cultures, stably transfected with the indicated constructs, throughout this study. In the presence of 25 mM of the hypoxia-mimetic CoCl₂, which resulted in robust HIF-1α stabilization (Fig EV1A), intense fluorescence was induced in both dUnaG- and dEGFP-expressing cells, demonstrating the functionality of the sensor constructs (Fig 1B). In clear contrast, only the UnaG-based sensor displayed an induction of fluorescence under hypoxia, which was induced in cultures that were grown at 1% oxygen.

In both cases, culture in the presence of 25 mM CoCl₂ or at 1% oxygen, we noted an increase in the mean fluorescence intensity between 12 and 24 h of incubation, suggesting that only after 24 h, the system had reached an equilibrium between synthesis and degradation (Fig 1C). Consistent with our previous results, dEGFP failed to show fluorescence enhancement under hypoxia. The spectral properties of dEGFP and dUnaG were largely comparable, with dUnaG displaying a wider emission spectrum (Fig EV1C). We were therefore able to observe dUnaG- and dEGFP-expressing cells side by side in different wells of the same chamber slide, which allowed us to use the same acquisition settings and readily replicated the flow cytometric analysis using live cell microscopy (Fig 1D).

In cultures grown under increasing oxygen tension, we observed decreasing dUnaG activation when the oxygen concentration was raised to 5%, while again dEGFP did not provide an enhanced fluorescence signal at all the oxygen concentrations tested (Fig 1E). UnaG fluorescence depends on non-covalent binding to its cofactor bilirubin (Kumagai et al., 2013). In tissue culture, bilirubin is supplied as a serum constituent in the medium, in our culture conditions at 2 µg/ml. We assessed UnaG fluorescence under different serum concentrations ranging from 0.5% to 11% (v/v) of the culture medium. After 24-h culture under hypoxia, UnaG fluorescence showed no further increase at serum concentrations higher than 5% (v/v) FCS, which corresponds to a 3.5 µM concentration of bilirubin (Fig 1F). In summary, our results demonstrate the usability of a destabilized version of UnaG to design an efficient genetically encodable hypoxia sensor.

Tuning of UnaG destabilization generates kinetically diverse sensors

Kinetic properties of a fluorescent sensor are essentially determined by the lifetime of the fluorescent protein. To design hypoxia sensors with different properties, we created variants of the basic UnaG sensor, in which UnaG is destabilized by fusion to a PEST sequence, the oxygen-dependent degradation domain (ODDD) of HIF-1α or both (Fig 2A). We compared the behavior of these sensor variants in long-term live cell imaging experiments, in which we acquired...
Figure 1. An UnaG-based hypoxia sensor efficiently indicates hypoxic growth conditions in CHO cells.

A Schematic representation of sensor constructs for the hypoxia-inducible expression of fluorescent proteins. Five HRE sequence motifs derived from the enhancer region of the VEGFA gene confer hypoxia-specific activity upon the CMV minimal (mCMV) promoter. Fluorescent proteins are destabilized by in-frame fusion of a PEST sequence from mouse ornithine carboxylase gene. The EGFP-encoding construct was obtained from Addgene (#46926).

B-D Comparative characterization of CHO cell bulk transfection cultures, selected for stable expression of the dUnaG- or dEGFP-encoding reporter constructs. Flow cytometry (B) revealed an efficient induction of green fluorescence, after the treatment of both dUnaG- and dEGFP-expressing CHO bulk cultures with CoCl$_2$, while growth under hypoxia (1% oxygen) for 12 h selectively induced green fluorescence only in dUnaG-expressing cells. Under normoxia (21% oxygen), only background fluorescence was observed. (C) Assessment of the mean fluorescence intensity (MFI) to determine the activity of the 5x HRE-CMV promoter after 12 and 24 h. (D) Representative images of dUnaG- or dEGFP-expressing CHO cells grown under either normoxia (21% oxygen), CoCl$_2$ treatment, or hypoxia (1% oxygen) for 12 h. Scale bars, 50 μm.

E Fluorescence intensity in dUnaG- or dEGFP-expressing CHO cell bulks grown under increasingly hypoxic conditions of 10%, 5%, and 1% oxygen for 24 h indicated an efficient induction of the dUnaG sensor at oxygen concentrations below 5%.

F Maximal fluorescence of the dUnaG-based hypoxia reporter in CHO cells grown at 1% oxygen for 24 h was only observed at serum concentrations of 5% FCS or higher, reflecting the dependence of UnaG on its serum cofactor bilirubin.

Data information: For flow cytometric measurements, all experiments represent 3 independent repeats. Bars show averages ± SE. MFI, mean fluorescence intensity.
Native UnaG (without any destabilization sequence) displayed a higher background fluorescence compared to destabilized forms, reflecting the protein half-life and leakiness of the 5x HRE-mCMV promoter (Fig 2D). In addition, fluorescence intensity was mildly increased even after reoxygenation, due to its high protein stability (Fig 2B and D). We determined a half-life of approximately 20.0 h for UnaG after shift to normoxic conditions (Fig EV2A). In comparison, background fluorescence was significantly lower for dUnaG and further reduced for the oUnaG sensor, where ODDD-mediated destabilization is driven by oxygen (Fig 2B). Background fluorescence was undetectable for the doubly destabilized doUnaG sensor. In the same order, the proteins showed an increasingly shorter life-time after reoxygenation with a half-life of 7.8 h for dUnaG, 5.5 h for oUnaG, and 30 min for doUnaG (Figs 2B and C, and EV2B). As

Figure 2. UnaG-based hypoxia sensor kinetics can be tuned by fusion to different protein destabilization domains.

A Schematic representation of the different variants of UnaG used in the four indicated hypoxia reporter constructs. dUnaG is identical to Fig 1A, oUnaG is destabilized by in-frame fusion with the oxygen-dependent degradation domain sequence (ODDD) from HIF-1α (aa 338–608), and doUnaG is destabilized by both degradation motifs.

B Microscopic assessment of the averaged fluorescence intensity (AFI) of CHO cell bulk cultures stably expressing the indicated UnaG variants or destabilized EGFP. Expression under hypoxia was induced by incubation in 1% oxygen for 16 h, and then, culture was continued for 24 h under normoxia. Fluorescence hysteresis after switching to normoxia was markedly reduced in the singly destabilized UnaG variants, while the doubly destabilized doUnaG displayed the most rapid switching behavior, but the least brightness. To reduce phototoxicity, illumination intensity was kept minimal. Data points represent one viewfield of 425 µm² ± SEM.

C Flow cytometric analysis of the induction and decay kinetics of the fluorescence intensity in CHO cells stably expressing either dUnaG or oUnaG. Open bars represent control cultures grown under normoxia. Solid bars indicate culture for 4, 8, 12, 16, 20, or 24 h under hypoxia (1% oxygen). To assess deactivation, cultures previously kept under hypoxia (1% oxygen) for 24 h were shifted to normoxia (21% oxygen) for the indicated times. Error bars represent SE.

D Representative maximum intensity projections (MIPs) from the life cell cultures depicted in (B). Cells were grown for the indicated times at hypoxic (1% oxygen) conditions and subsequently switched for the indicated times to normoxia (21% oxygen) (red arrows in B) to illustrate the dynamics of the different UnaG-based hypoxia sensors. Increasing destabilization is associated with reduced brightness but also reduced background and improved switching behavior. Scale bars, 50 µm.
proximity to blood vessels, which we interpreted as an indication of
interred in poorly vascularized areas (Fig 3A and B). Of note, we
had observed immunostaining. We were positively surprised by the brightness of
UnaG-expressing cells with different switching kinetics can be produced, which
will retain memory of hypoxia in a designable fashion.

The hypoxia sensor dUnaG faithfully marks cells with stabilized,
uclear HIF-1α in vivo

We wondered whether the UnaG-based hypoxia sensors were appli-
cable in vivo. To test their suitability to visualize hypoxia in tumor
tissue, we stably transfected mCherry-expressing Gli36 human
glioblastoma cells with the dUnaG sensor construct. We recovered
the developing tumors and surrounding cortex 10 days after
orthotopic transplantation into SCID mice and prepared thick
(30-μm) sections for analysis. We identified blood vessels by
PECAM-1 staining (Fig 3A). Within the tumor tissue that was
suffusedly demarked by mCherry expression, the green fluorescence of
dUnaG was readily detected without the need for additional
immunostaining. We were positively surprised by the brightness of
dUnaG in sections, which exceeded the fluorescence intensities,
we had observed in vitro. As expected, UnaG was preferentially clus-
tered in poorly vascularized areas (Fig 3A and B). Of note, we
frequently observed individual dUnaG-expressing cells in close
proximity to blood vessels, which we interpreted as an indication of
cells relocating from hypoxic areas (Fig 3B).

Immunostaining confirmed the expected preferential nuclear
localization of stabilized HIF-1α and revealed that > 95% of the
dUnaG-expressing cells also stained for nuclear HIF-1α (Fig 3C and
D). We noted that cells expressing dUnaG to a large fraction
(> 60%) displayed reduced mCherry fluorescence at or below
background level, which we interpreted as the consequence of
impaired mCherry maturation due to a hypoxic environment
(Fig 3C and D).

Being genetically encoded, the dUnaG reporter should be
perfectly suited for intravitral imaging. To test its principal applica-
ble, we recorded the multiphoton excitation spectrum of dUnaG in
stably transfected CHO cells and determined the optimal wavelength
of excitation to be between 800 and 820 nm (Fig EV1C and D).
Having established the parameters for multiphoton imaging, we
orthotopically transplanted Gli36 cells stably transfected with a
mCherry expression vector and dUnaG into SCID mice, to which a
cranial window had been applied.

In accordance with the postmortem analysis, dUnaG-expressing
cells were readily detected as clusters in sparsely vascularized
tumor regions (note hypoxic islands bounded by red line in Fig 3E).
In maximum intensity projections (MIPs) (Fig 3E, bottom right
panel), we noted an apparent overlap between sites of hypoxia and
blood vessels. To resolve this aspect, we performed depth color
coding, which revealed a significant axial distance between vessels
and hypoxic cell clusters. In Fig 3F, both hypoxic islands are located
toward the top of the imaged tissue volume, indicated by the
predominance of purple, red, and yellow-green colors in the depth
color coding. The two large, perfused vessels at lower edge (see
Fig 3E Qdots, depicted in turquoise) are located at the bottom of the
imaged tissue cube (indicated by preferentially dark blue and azure
depth coding). The depth color coding also showed that the
mCherry-positive cells within the bounded hypoxic region at the
bottom right are located at the bottom of the imaged tissue cube
(indicated by preferentially dark blue depth coding). Hence, these
tumor cells reside underneath the hypoxic cap and are flanked by a
large perfused vessel, which likely provides sufficient oxygen to
upkeep the residual mCherry signal.
Figure 3.
UnaG-based genetically encoded hypoxia sensors

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Taken together, we demonstrate here the applicability on the dUnaG sensor in vivo and provide evidence that this sensor marks hypoxic areas in progressing tumors.

A d(UnaG-mOrange) fusion protein can be employed as a hypoxia–reoxygenation sensor to reveal cells with a recent hypoxic history

We hypothesized that a combination of the unique oxygen-independent and oxygen-dependent maturation properties of UnaG and mOrange should allow the design of a sensor that reports the recent hypoxic history of cells and displays oxygen levels at cellular resolution. To this end, we designed and evaluated a number of sensor constructs (Figs 4 and EV3). Here, we describe the characterization and application of the sensor construct dUnOHR, comprising an in-frame fusion protein of UnaG and mOrange, which is destabilized by an ornithine decarboxylase PEST sequence (Fig 4A).

The nomenclature dUnOHR indicates the fusion of UnaG and mOrange as well as the intended use of this construct under hypoxia–reoxygenation conditions.

We confirmed the expected behavior of dUnOHR in live cell imaging experiments, in which we recorded both UnaG and mOrange fluorescence. To minimize phototoxicity during the long-term measurement, we used the minimal possible excitation light intensity, which precluded the use of online fingerprinting. Because of the broad emission spectrum of UnaG, we excited mOrange at 561 nm and only collected fluorescence emission at wavelengths > 600 nm. This minimized bleed through from UnaG, but sacrificed a considerable amount of mOrange signal. In cultures grown under normoxia, where the promoter is inactive, dUnOHR produced virtually no background signal (Fig 4B and C, Video EV2). During repetitive hypoxia–normoxia cycles, we observed the expected increase in the UnaG signal under hypoxia, while fluorescence of the mOrange moiety remained undetectable. Following reoxygenation, a limited increase in UnaG fluorescence preceded promoter shutdown and decay. Simultaneously, the mOrange chromophore matured and mOrange fluorescence became detectable and subsequently also decayed. This behavior repeated in a subsequent hypoxia–reoxygenation cycle (Fig 4B and C, Video EV2). The higher signal increase in the second reoxygenation cycle in comparison with the first one is due to the proliferation of cells in the live cell culture.

The unique properties of the dUnOHR sensor allow the discrimination of hypoxic and reoxygenated cells. Hypoxic cells display only UnaG fluorescence, while recently reoxygenated cells display the both UnaG and mOrange fluorescence.

Visualization of the oxygenation history with dUnOHR suggests frequent movement of cells from hypoxic to reoxygenated areas

To take advantage of the unique properties of dUnOHR in vivo, we orthotopically transplanted Gli36 cells stably transfected with this sensor into SCID mice. We recovered the developing tumors and adjacent tissue 10 days later and generated 30-μm sections for analysis. As previously observed for the dUnaG sensor, the dUnOHR sensor marked clusters of cells in hypervascularized areas, which were identified by sparse or absent PECAM-1 immunostaining (Fig 5A–C). The majority of these cells only displayed UnaG fluorescence. A fraction of cells in addition expressed mOrange, and very frequently, such cells were found at the border of UnaG-positive clusters, often seen in proximity to the nearest blood vessels. We interpreted these observations as a sign of recent relocation into the reoxygenation zone at the border of the hypoxic area. This may suggest that a fraction of hypoxic cells display a tendency to emigrate from the hypoxic environment toward areas of higher oxygen tension (Fig 5B and C).

At the edges of hypoxic clusters, we frequently observed intensely fluorescing mOrange spots. Imaging at high resolution revealed that cells, which harbor these spots, also displayed signs of nuclear and cytoplasmic fragmentation indicative of an apoptotic fate (Figs 5D and EV4). To confirm this assumption, we performed
Figure 5. Preferential localization of cells that have undergone recent reoxygenation to the edge of hypoxic areas.

A Overview of a tumor induced by deep cortical deposition of approximately 500 gli36 cells stably transfected with the dUnOHR sensor construct. The perimeter of the developing tumor (white line in the merged picture, lower right panel) was delineated by Hoechst 33342 staining. As observed for dUnaG, the reoxygenation sensor was expressed in compact areas with low vascular density. Vascular endothelium was identified by PECAM-1 staining. 30-µm thick cryosection. Scale bar, 500 µm.

B Details of the tumor shown in (A). Sparse, distinct cells are characterized by intense green and orange fluorescence (shown as yellow in the merged panel) and are located preferentially to the edge of the hypoxic phalanx (white arrows). A number of cells in close proximity of the nearest blood vessel display weak green and orange fluorescence (asterisks), indicative of previously hypoxic environment and subsequent reoxygenation. Scale bar, 50 µm.

C Recently reoxygenated cells (arrows) cluster at the edge of hypoxic areas and are often detected in the proximity of vessels with a homogeneous caliber (double arrows). Scale bar, 100 µm.

D Previously hypoxic cells frequently undergo apoptosis after reoxygenation. We noted an accumulation of intensely orange fluorescing spots (white arrows) at the edges of compact UnaG-positive areas. Scale bar, 50 µm.

E TUNEL staining of tumor sections revealed that the tissue areas rich in intense mOrange aggregates colocalized with areas of increased apoptosis. Scale bar, 50 µm.
TUNEL staining on tissue sections and found a pronounced colocalization between cells rich in orange spots and TUNEL-positive cells (Fig 5E).

Taken together, we demonstrate here that the dUnOHR sensor can be used in vivo and allows, in addition to a visualization of hypoxic cells, the identification of cells that were recently reoxygenated.

Discussion

We describe here a novel family of sensor constructs for the visualization of tissue hypoxia in light microscopy. These sensors are based on a HIF-inducible minimal CMV promoter driving the expression of destabilized versions of the fluorescent protein UnaG, which does not rely on molecular oxygen for its fluorescent maturation (Kumagai et al., 2013; Mishin et al., 2015). These sensor constructs are genetically encodable and hence do not rely on the administration or tissue penetration of reagents. Because the spectral properties of UnaG are very close to those of the widely used Aequorea GFP, this sensor family is compatible with existing wide-field fluorescent microscopes and is also applicable for intravital approaches using multiphoton microscopy.

Through tuned destabilization of the lifetime of the fluorescent reporter UnaG, switching kinetics were adjusted, which allows the design of reporters for various tasks, ranging from constructs with long memory to rapid visualization of changing tissue oxygen levels.

Two observations made us rather confident that this reporter approach truly detects hypoxic cells in vivo. First, activation of our reporter constructs coincided with the stabilization of nuclear HIF-1α in vitro and in vivo. Second, cells that expressed the reporter displayed a significantly reduced fluorescence of coexpressed mCherry, indicative of impaired chromophore stabilization due to an insufficient oxygen tension. While hypoxia-independent activation of HIF-1α signaling has been described, orchestration of the hypoxia response appears to be the central and by far predominant function of this transcription factor family.

In tissue culture, our sensor constructs became first detectable around 5% oxygen and reached full activity at 1% oxygen, which corresponds to 7.5 mm Hg and coincides well with the reported threshold of 10 mm Hg for the induction of a tissue hypoxia response (Hockel & Vaupel, 2001; Span & Bussink, 2015).

Acquisition of the fluorescent state of UnaG depends on non-covalent binding of its cofactor bilirubin (Kumagai et al., 2013). In tissue culture, bilirubin is supplied as a constituent of fetal calf serum (FCS). In the absence of bilirubin-depleted serum, we varied the FCS concentration in the growth medium and consequently the bilirubin concentration. UnaG fluorescence remained stable from 5% or higher FCS reconstitution. We cannot preclude an influence of serum concentrations less than 5% on UnaG biosynthesis, due to the overall reduced cellular activity. However, increasing serum concentrations beyond 5% FCS did not result in a proportional increase in UnaG fluorescence, suggesting that a concentration of 5% FCS that corresponding to 3.5 μM bilirubin suffices for maximal UnaG fluorescence.

In our in vivo experiments, we readily detected UnaG fluorescence in glioblastoma samples without enhancement by immunostaining or provision of exogenous cofactor, suggesting that despite the blood–brain barrier, sufficient bilirubin is ubiquitously provided by the circulation or locally.

Sensors combining oxygen-dependent and oxygen-independent proteins will allow the design of probes with enhanced functionalities, like ratiometric sensors to measure absolute oxygen levels in tissue. We have taken a first step in this direction with the design of dUnOHR, which reports the recent hypoxic history of cells. Our observations suggest that upon reoxygenation previously hypoxic tumor cells display a propensity to undergo apoptotic cell death. Hypoxia-induced apoptosis is well described and has previously been reported to rely on HIF-1-dependent, but also HIF-1-independent genes (Carmeliet et al., 1998; Malhotra et al., 2001).

We frequently observed a small fraction of reoxygenated cells in proximity to blood vessels, indicative of their migration from a hypoxic zone. This interpretation is supported by previous reports demonstrating that hypoxia enhances the metastatic potential of breast cancer cells into bone marrow and HIF-1α downregulation by siRNA reduces glioma cell invasiveness in various tumor models (Fujiwara et al., 2007). The speed of slowly migrating large polarized cells in 3D matrices was determined to be around 0.5 μm/min (Friedl et al., 1998). Given the maximum mOrange fluorescence in our reoxygenation reporter being observed around 4–6 h after reoxygenation (Fig 4B), this would amount to a migration distance of 120–180 μm, which fits well to the distances observed in Fig 5B and C. Interestingly, hypoxia has also been suggested to induce vascular remodeling by promoting the migration of vascular smooth muscle cells (Revuelta-Lopez et al., 2013). An alternative interpretation for the presence of the reoxygenated cells in proximity to the blood vessels would be due to the transient vessel occlusion (Janssen et al., 2002).

The importance of hypoxia as a driving force in tumor vascularization and likely tumor metastatic spread is well accepted. We believe that the possibility to directly visualize tissue hypoxia at cellular level by light microscopy will be a valuable addition to the existing possibilities to investigate the role of hypoxia not only in tumors but also in other important tissues and biological processes.

Many of the central questions concerning the fate of hypoxic cells and their migratory behavior now become accessible through intravital microscopy. Transgenic mouse models expressing UnaG-based hypoxia sensors in an organ-specific or inducible fashion should become invaluable tools for these investigations.

Materials and Methods

Plasmid design

Plasmid containing 5 HRE sequences in tandem to a minimal CMV promoter was obtained from Addgene (#46926) (Vordermark et al., 2001). This plasmid was digested with NcoI and NotI enzymes (NEB) to release the d2EGFP insert fragment. UnaG was PCR-amplified with overhang primers that contain a MCS in its N-terminal and inserted into the HRE-containing vector using the NcoI and NotI sites. PEST sequence was amplified from d2EGFP (Addgene #26164). ODDD region from HIF-1α (Addgene #18949) was amplified (AA338-608) (Chan et al., 2005), so as to contain prolines 402 & 564. mOrange was
amplified from pcDNA3.1-mOrange plasmid. The P2A sequence (GGCAGTGGAGAGGGCAGAGGAAGTCTGCTAACATGCGGTGACGT CGAGGAGAATCCTGGCCCA) was amplified from plasmids made for other works. All the PCR-amplified products were restriction-digested and cloned sequentially into the MCS region.

Cell lines

Stable cell lines were made in CHO and Gli36 cells. Cells were cultured at 37°C with 10% CO2 in DMEM supplemented with 10% FCS (Biowest Cat.# S1800), 100 μg/ml penicillin and streptomycin, and 2 mM glutamine. For experiments requiring 1% oxygen (hypoxia), cells were placed in an oxygen-regulated incubator (HeraCell-150, Thermo scientific). For hypoxia experiments on the microscope, a gas mixer was used to pump gas with 1% oxygen, 10% CO2, and rest of nitrogen to the sample-holding chamber. NanoJuice transfection kit (Millipore-71902) was used for transfections. All transfections were done according to the NanoJuice manufacturer protocol. A day after the transfection, cells were placed under selection with G418 sulfate (GIBCO) for two weeks, after which they were sorted based on UnaG fluorescence.

Cytometry

BD FACS Canto was used for cell analysis, and BD FACS Aria was used for cell sorting. In the process of making stable cells, the sensor-transfected cells were sorted following chemical selection. Chemically selected cells were placed in 1% oxygen for 12 h prior to sorting. Cells were trypsinized and resuspended in DMEM supplemented with 10% FCS and antibiotic. All UnaG-positive cells (FITC positive) were sorted into a collection tube with 2 ml of media. For cell analysis, cells were trypsinized and resuspended in PBS with 3% FCS. Flow cytometry data were exported as FCS files and analyzed using FlowJo software.

Microscopy

Zeiss LSM 780 microscope was used for live cell time-lapse imaging and imaging of immunostained samples. The microscope is equipped with a humidified incubation chamber attached with a gas mixer. To enable better quantification when comparing different sensor constructs, cell lines were plated in different wells of the same IBIDI Treat (80826) 8-well chamber slide. Position scanning module was used to acquire various sensor variants in the 8-well slide. UnaG was excited at 488 nm, and mOrange was excited at 561 nm. The excitation laser intensity was always kept low to avoid photodamage to the cells. For time series experiments, images were acquired at an interval of 5 min. For in vivo imaging via cranial window, a LaVision Biotech multiphoton microscope (TriMScope) was used. This system is equipped with two tunable mode-locked Chameleon XR Ti:Sapphire lasers. One of the lasers is used to pump an optical parametric oscillator (OPO) that generates wavelengths greater than 1,000 nm. UnaG was excited at 820 nm and mCherry was excited at 1,100 nm simultaneously. Mice were anesthetized and placed on a heated chamber while imaging. Blood vessels were visualized by Qtracker 705 Vascular Labels (Life technologies—Q21061MP), injected via tail vein prior to imaging. The Qtracker labels were excited at 820 nm.

Cranial window & intracranial injection

Fifteen-week-old male SCID mice were used for all in vivo experiments. Cranial window was done as described earlier (Holtmaat et al., 2009), with one additional tumor cell injection step done before closing the window. Stable sensor-transfected Gli36 cells were used for injections. Cells were trypsinized and resuspended in DMEM without FCS at the concentration of 50,000 cells/ml. Two microliters of this cell suspension was injected using a Hamilton microneedle, before sealing the cranial windows with a glass coverslip. The mice were imaged every day following injection. Tumor cells could be seen starting 3 days after injection. For obtaining tissue for immunostainings, tumor cells were intracranially injected into SCID mice as described earlier (Baumann et al., 2012). Two weeks after the injection, mice were euthanized following PFA perfusion. Subsequently, the brain was explanted and cryosectioned to obtain tissue slides.

Immunohistochemistry

A proprietary PECAM antibody was used to stain blood vessels. HIF-1α was obtained from GeneTex, Inc (Gene Tex-GTX127309). The tissue sections were washed in PBS (3×) for 10 min. The slides were permeabilized with 0.1% Triton X-100 in 1% BSA/PBS for 1 h. Primary antibody was allowed to bind in 1% BSA/PBS for 2 h, followed by washing with block buffer for (3×) 10 min. Secondary antibody was incubated for 30 min. The tissue sections were sealed with a glass coverslip using mounting media. TUNEL staining was done using Click-it® TUNEL Alexa Fluor® 647 kit according to the manufacturer protocol.

Image analysis

All image analysis was done using Fiji. For processing the live cell time-lapse data, the time series analyzer plugin was used. The z-stacks obtained from the time series were combined using the maximum intensity projection function. The entire image was divided into 25 rectangular regions using a custom ImageJ plugin, and the intensity across the time was obtained from all ROIs using time series analyzer. The data were saved in a csv file and analyzed by R. All the immunostained images were z-projected with a maximum intensity for presentation. When required, to reduce the noise, mean filtering with a filter radius of 2 was applied.

Statistical analysis

Statistical programming software R was used for data processing and plotting. All plots were done with ggplot2 graphics package. Standard deviation or error was plotted as indicated in the figures. Standard deviation was calculated using the sd function from the base package, and the standard error was calculated using the standard error from the plotrix package. Curve fitting tool box from Matlab was used to fit exponential distribution and calculate the half-lives.

Expanded View for this article is available online.

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Author contributions

RE and FK designed the experiments, evaluated the results, and wrote the manuscript. RE performed all the experiments and analysis. WB and MS provided reagents and support during the project.

Conflict of interest

The authors declare that they have no conflict of interest.

References


